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Review

Recent investigations into the lipoxygenase pathway of plants

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Key words: Lipoxygenase pathway; Hydroperoxide product; Plant enzyme; Enzyme pathway; Fatty acid hydroperoxide

Contents

Abbreviations: LOX, lipoxygenase; HPDS, hydroperoxide dehydrase; HPLS, hydroperoxide lyase; HPIS, hydroperoxide isomerase; HPEP, hydroperoxide-dependent epoxygenase; HPPR, hydroperoxide-dependent peroxygenase; 13*S*-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13*S*-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 9*S*-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 9*S*-HPOT, 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; NDGA, nordihydroguaiaretic acid; 5*S*-HPETE, 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid; 15*S*-HPETE, 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 12*S*-HPETE, 12(*S*)-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; EPR, electron paramagnetic resonance; 8*R*-HPETE, 8(*R*)-hydroperoxy-5(*Z*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; 12,13*S*-EOD, 12,13(*S*)-epoxy-9,11-octadecadienoic acid; kDa, kiloDaltons; 13*R*-HPOD, 13(*R*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 9*R*-HPOD, 9(*R*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid.

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I. Summary

The plant lipoxygenase (LOX) pathway is in many respects the equivalent of the 'arachidonic acid cascade' in animals. The LOX-catalyzed dioxygenation of the plant fatty acids, linoleic and linolenic acids, is followed by metabolism of the resulting fatty acid hydroperoxides by other enzymes. Although the physiological functions of the end-products do not appear to be fully defined at this time, hormonal and anti-fungal activities have been reported.

II. Introduction

Lipoxygenase (LOX) is distributed throughout the plant kingdom and may be present in all plants. LOX catalyzes the oxygenation of the (*Z*),(*Z*)-pentadiene moiety of polyunsaturated fatty acids into enantiomeric hydroperoxide fatty acids with (*Z*),(*E*)-diene conjugation. Although the predominant polyunsaturated fatty acids of plants are generally linoleic and linolenic acids, many other fatty acids having the requisite structure also can serve as substrates.

An array of plant enzymes metabolize the hydroperoxide products of LOX, such as hydroperoxide dehydrase (HPDS), hydroperoxide lyase (HPLS), hydroperoxide isomerase (HPIS), hydroperoxide-dependent peroxygenase (HPPR) and hydroperoxide-dependent epoxygenase (HPEP). And, LOX itself is capable of converting fatty acid hydroperoxides into a variety of compounds. Until recently, it appeared as if hydroperoxide-active enzymes of plants and animals were distinctly different, but examples of comparative biochemistry are becoming common.

Although the structure of a number of interesting hydroperoxide metabolites has been defined in detail, an explanation of their physiological function appears to be less than complete with notable exceptions, such as the plant hormone, jasmonic acid and several antifungal substances. The many physiological activities known for products of the animal LOX pathway would appear to predict similar crucial roles for hydroperoxide metabolites in plants.

This review focuses on recent research (last 10 years) on the enzymic reactions of LOX-generated fatty acid hydroperoxides. Also, recent advances into understanding the physiological significance of the LOX pathway in plants are discussed. The review of plant LOXs per se mainly relates to its role in the pathway, as well as its mode of action.

III. Lipoxygenase

There is an enormous literature regarding plant LOXs, including at least 50 reviews on the subject. The following abbreviated list of reviews is recommended for their more comprehensive coverage concerning historical perspective [1]; literature prior to the mid 1970s, regarding general aspects [2,3] and food quality [4]; research preceding the early 1980s [5–7]; and recently, general topics [8], cereal LOXs [9] and mechanistic studies [10]. Most of the above citations also include discussion of enzymes that utilize hydroperoxides as substrates.

Soy LOX-1, considered to be a model, has been the most thoroughly characterized of the LOXs. The crystallization of this enzyme was reported by Theorell and co-workers in 1947, but progress was relatively slow for the next 20–25 years. After H.W.-S. Chan announced the presence of one mole of iron per mole of soy LOX-1 at the 1972 meeting of the International Society for Fat Research, LOX research seemed to expand exponentially. Currently, the primary structure of LOX-1 has been fully determined setting its molecular weight at 94038 [11], a size approximately equivalent to most plant LOXs. In addition, two other isozymes from soybean seeds have been cloned and sequenced [12,13] permitting comparisons of homology among all three isozymes [12]. The soy isozymes, as well as pea LOX, have interesting homologies with animal LOXs, possibly indicating conservation of sequences at the active site and a fatty acid binding site [10,14]. Preliminary X-ray analyses of soy LOX-1 crystals promises to eventually resolve its tertiary structure [15,16]. In addition to the three isozymes originally thought to be present in soybean seeds, at least one additional isoform has been found [17–19] and other separate isozymes were found in germinating seeds [20] and leaf tissue [19].

Plants other than soybean have been shown to express isozymes. For example, two of the three pea seed LOX isozymes recently have been cloned and sequenced [21,22]. According to Domoney et al. [23], peas had at least seven isozymes, five of which were minor. Isozymes have been recently demonstrated in potato [24], Bengal gram [25] and maize [26]. Other examples of isozymes are cited in earlier reviews [4,8,9].

By 1967 Hamberg and Samuelsson had made a significant advance into understanding the regio- and stereo-specificity of product formation by soy LOX-1. They found that an ω-8 methylene hydrogen was removed stereospecifically (e.g., the pro-(*S*) hydrogen of linoleic acid) in a rate-limiting step on the opposite side of the fatty acid molecule relative to O_2 insertion at the ω-6 carbon (*S*-stereospecific, *L* by the Fischer convention). This oxidation specificity proved to be typical of many plant LOXs and with the common plant fatty acids, linoleic and linolenic acids, oxidation resulted in the formation of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HPOD) and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HPOT), respectively. With the eicosanoid fatty acids, which actually are exotic to plants, the

oxidation products were usually 15(*S*)-hydroperoxides. Although the preferred substrate of soy LOX-1 is free fatty acids, it was recently reported that fatty acids acylated to phospholipids are slowly oxygenated at the ω-6 carbon with *S*-stereoconfiguration [27].

Two decades ago, another class of LOXs were found in potato tubers and maize kernels, in Galliard's and Gardner's laboratories, respectively. These LOXs effected oxygenation of linoleic acid and linolenic acid into 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9*S*-HPOD) and 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9*S*-HPOT), respectively (*D* by the Fischer convention). Like soy LOX, both hydrogen removal from C-11 methylene [pro-(*R*)] and O_2 insertion occurred on opposite sides from one another, but with completely opposite orientation compared to soy LOX-1 [28]. With this type of LOX, arachidonic acid was mainly oxidized into 5(*S*)- and 8(*S*)-hydroperoxides [24,29]. Unlike the soy LOX-1 type, which recognized the ω-6 position, these LOXs apparently used the carboxylic acid group as a signal [29].

Interestingly, soy LOX-1 has been found to be unique in that it catalyzed the oxidation of linoleic acid into both 13*S*-HPOD and 9*S*-HPOD at pHs less than 9. The negligible percentage of 9*S*-HPOD produced at pH > 9 was steadily increased to about 25% at the expense of 13*S*-HPOD by lowering the pH to about 6. This transformation occurred in parallel with the linoleate to linoleic acid titration implying that the carboxylic acid moiety was a prerequisite for oxygenation at C-9 [30]. An analogous effect of pH on the oxidation of γ-linolenic acid into 9- and 13-hydroperoxides was also observed [31]. In order for the enzyme to catalyze either pro-(*S*) or pro-(*R*) hydrogen removal from C-11 and O_2 insertion with (*S*)-stereospecificity at either C-13 or C-9, spatial identity at the active site was proposed to be achieved by a head-first or tail-first orientation of linoleic acid (Fig. 1a). As shown in Fig. 1b, the conversion of the unusual substrates 9(*Z*),12(*E*)- and 9(*E*),12(*Z*)-octadecadienoic acids into 13(*R*)- and 9(*R*)-hydroperoxides, respectively, again demonstrated the stereochemistry of head to tail orientation [32]. However, this specificity of soy LOX-1 was apparently not shared by some other LOXs, like those from pea [33] and potato [34] which formed major and minor hydroperoxide products with predominantly (*S*) and (*R*) stereoconfiguration, respectively. Another class of LOXs, exemplified by soy LOX-2 at neutral pH values [33] and pea LOX-1 [35], furnished hydroperoxides that were less stereochemically pure and consequently, may be more peroxidative in their mode of action.

Research has been intense concerning the nature of the LOX active site using soybean LOX-1 as the model. Excellent reviews are available on this subject

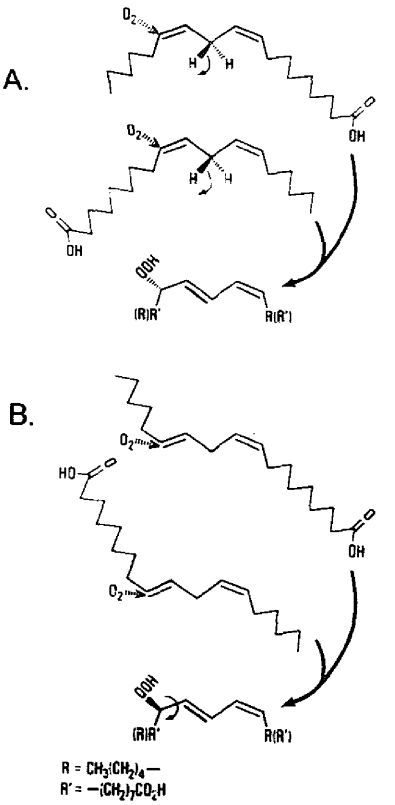


Fig. 1. (a) Oxidation of linoleic acid by soy lipoxygenase-1 to either the 9(*S*)- or 13(*S*)-hydroperoxide is spatially identical when the substrate is arranged head to tail in opposite orientations. (b) According to Funk et al. [32], the unusual substrates, 9(*Z*),12(*E*)- and 9(*E*),12(*Z*)-octadecadienoic acids, are also oxidized in opposite orientations furnishing 9(*R*)- and 13(*R*)-hydroperoxides. Reprinted from Ref. 30.

[6,7,10,36]. The native-resting enzyme, a relatively inactive form, is high-spin ferrous [36–38], which is converted to the active ferric form (yellow enzyme) by oxidation with either product hydroperoxide [36,39], $<$ nM concentrations of H_2O_2 [40], or possibly even atmospheric O_2 catalyzed by the impurities in dilute solutions of LOX-1 [41]. After activation, LOX-1 functions by a redox cycle between high-spin ferric and high-spin ferrous as fueled by substrate and fatty acid peroxyl radical [10,36,42,43]. The ferric enzyme is responsible for oxidizing the fatty acid (*Z*),(*Z*)-1,4-pentadiene moiety to the pentadienyl radical, which in turn reduces ferric to ferrous. The actively cycling reduced enzyme is high-spin ferrous, like the native enzyme, but the former is distinct, possibly by virtue of its binding to the substrate pentadienyl radical [42]. The pentadienyl radical is thought to react with molecular O_2 to afford a peroxyl radical and the cycle is completed by

reduction of peroxyl radical with ferrous to give peroxyl anion and the activated ferric form of the enzyme. LOX also participates in a peroxidative cycle caused by anaerobic conditions; that is, lack of oxygen results in redox cycling by its substrate and hydroperoxide product with release of pentadienyl and alkoxyl radicals. Fig. 2 shows one of a number of schemes that have been proposed for the aerobic and anaerobic functions of LOX (e.g. Refs. 2,8,9,36,44), all of which are basically similar to the one reported by de Groot and co-workers in 1975.

An interesting alternative model for LOX-1 has been proposed by Corey et al. [45–50], which involves direct insertion of O_2 into an organoiron intermediate facilitated by deprotonation with an enzymic basic moiety. On the basis of α-secondary isotope effects of substrate labeled with tritiated olefinic carbons, Wiseman [51] suggested that rather than direct O_2 insertion, the complex of organoiron with substrate leads to a substrate-carbanionic intermediate after proton abstraction. This intermediate was then proposed to be converted into a pentadienyl radical by electron transfer to iron. Although the involvement of a basic group seems certain based on suicide inactivation by specially designed substrates [48,49], several arguments have been made against the organoiron model. In view of the result with keto- [52] and fluoro- [53] substituted substrates, a carbanionic intermediate seemed unlikely. And, the detection of peroxyl radical by electron paramagnetic resonance (EPR) during the active cycling of LOX-1 appeared to refute the direct O_2-insertion organoiron model [54]. Further investigation showed that purple LOX-1, a 1:1 complex of LOX-1 with 13S-HPOD, favored the formation of peroxyl radical under conditions of O_2 saturation; whereas, depletion of O_2 from purple LOX-1 led to detection of pentadienyl radicals [43]. That peroxyl and pentadienyl radicals were bound as enzyme complexes mechanistically connected was indicated by their relatively long life and by exchange of atmospheric O_2 with both peroxyl radical [43] and the hydroperoxide group of added 13S-HPOD [55]. Such an equilibrium between peroxyl radical and pentadienyl radical plus O_2 is already known from research of the free radical chemistry of autoxidizing polyunsaturated lipids.

Much effort has been expended on probing the nature of the active site of LOX-1, particularly in regard to ligands of iron. Various spectral methods have indicated that the iron of LOX-1 was roughly octahedral six-coordinate [38,56] with rhombic distortion [56,57]. The ligands were nitrogen and oxygen [38,58], but not molecular O_2 [57,59]; non-complexation of molecular O_2 indicated that chiral insertion of O_2 was not completed by iron and that the iron active site solely activated substrate [59]. The nitrogen ligands appeared to be 4 ± 1 imidazoles [58] and at least one other ligand was occupied by H_2O [60]. The H_2O can be displaced by the exogenous ligand, nitric oxide, a

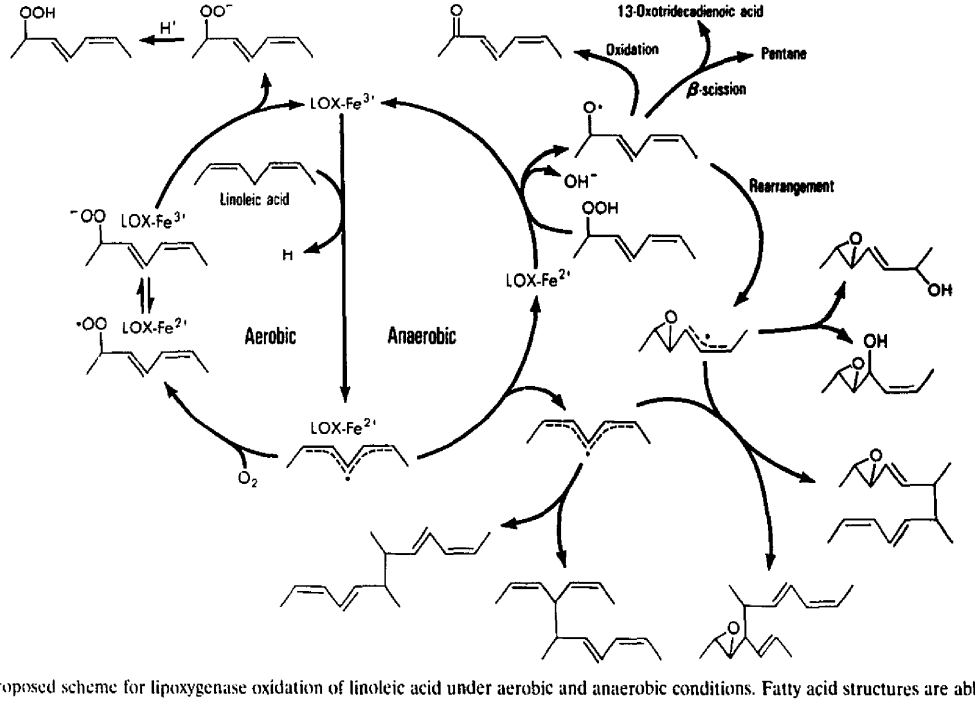


Fig. 2. Proposed scheme for lipoxygenase oxidation of linoleic acid under aerobic and anaerobic conditions. Fatty acid structures are abbreviated to show only C-8 through C-14. Modified from de Groot et al. 1975; reprinted from Ref. 9.

competitor of substrate binding [61]. Van der Meer and Duine [62] proposed that a previously unsuspected cofactor discovered by them, pyrroloquinoline quinone, was complexed to iron, but this was difficult to rationalize in view of the work described above. Indeed, two different laboratories [63,64] have recently reported that pyrroloquinoline quinone was not present in soybean LOX-1, including the other three LOX isozymes of soybean [64].

Specific inhibitors have been used to probe the active site of soy LOX-1. Among the more specific reversible inhibitors, the catecholates, affected the enzyme in two principal ways. First, catecholates with fewer electron-withdrawing substituents, like catechol [65] and nordihydroguaiaretic acid (NDGA), reduced the active ferric form of LOX-1 to the inactive ferrous state, apparently the native enzyme [66]. In turn, the catecholates were oxidized to their semiquinones [67]. Secondly, catecholates with electron-withdrawing substituents, like 4-nitrocatechol and 3,4-dihydroxybenzaldehyde, formed relatively stable complexes with LOX-1 and additionally did not cause reduction of the ferric enzyme [65,68]. It has been suggested that the catecholates occupy the exogenous ligand site, identical to the site that nitric oxide can occupy, as well as displace an endogenous ligand [68]. Like catechol and NDGA, other reversible inhibitors, *N*-octylhydroxylamine or *N*-decylhydroxylamine, inhibited LOX-1 by reducing the active ferric form [69]; inhibition was reversed by oxidizing with 13*S*-HPOD. Hydroxylamine itself did not serve as an inhibitor since the octyl or decyl groups evidently were required for delivery to the active site. The suicide inhibitors, 12-iodo-9(*Z*)-octadecenoic acid [70], colneleic acid [71], acetylenic fatty acids [72] and hexanal phenylhydrazone [73], were substrate mimics. The latter two were oxidized into reactive products by LOX which caused oxidation of essential methionine(s) at the active site.

In view of the recent progress with understanding the structure and function of LOX-1, it is certain a comprehensive view of the enzyme will soon be known.

IV. Secondary products from lipoxygenase reactions

LOX is versatile in catalyzing the formation of products other than monohydroperoxides. The formation of these so-called secondary products can be classified into the following reaction categories: (a) double dioxygenation into dihydroperoxides; (b) leukotriene and lipoxin formation; and (c) free radical reactions catalyzed by anaerobic cycling of LOX.

IV-A. Dihydroperoxides

In 1977 Bild and co-workers reported that soy LOX-1 catalyzed the double dioxygenation of fatty acids, such as γ-linolenic, arachidonic and all (*Z*)-8,11,14-eicosatrienoic acids; that is, fatty acids with an all (*Z*)-octatriene moiety located between ω-6 and ω-12 positions. The first dioxygenation rapidly occurred at ω-6 providing either 13(*S*)- or 15(*S*)-hydroperoxides with usual LOX-1 specificity; the second oxygenation was slower (200-fold higher K_m) and was promoted by lower pHs [6.8–8.7]. The dihydroperoxides of arachidonic acid were identified as 8(*S*),15(*S*)-dihydroperoxy-5(*Z*),9(*E*),11(*Z*),13(*E*)-eicosatetraenoic acid and 5(*S*),15(*S*)-dihydroperoxy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid in a 3:2 ratio [74]. The second dioxygenation step was reminiscent of the 9(*S*)-oxidation specificity of soy LOX-1 discussed above (Fig. 1a) and was similarly promoted by pHs lower than the 9–10 optimum required for the first ω-6 oxygenation. Thus, soy LOX-1 appears to double dioxygenate by the same head to tail recognition process previously described. Although Bild et al. did not observe dihydroperoxides of α-linolenic acid (the formation of 13*S*-HPOT precludes this possibility), Sok and Kim [75] succeeded in producing a 9,16-dihydroperoxide by lowering the pH to 6.5. At these low pHs a significant but minor portion of the primary products would presumably be 9*S*-HPOT, which then would oxygenate into the dihydroperoxide. A virtually identical result was obtained by incubating 9*S*-HPOT directly with soy LOX-1. As might be expected, the formation of dihydroperoxides from several polyunsaturated fatty acids was eliminated at pH 11, but not the ω-6 oxidation to monohydroperoxides [76].

Potato LOX, which utilizes the carboxylic acid moiety as a signal, produced the 5(*S*),12(*S*)-dihydroperoxide from either arachidonic acid [24] or 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5*S*-HPETE) [77]. The steric outcome of this double dioxygenation was similar to that of soy LOX-1; that is, the dioxygenation was antarafacial. On the other hand, under high O_2 tensions (60 atm) arachidonic acid was double dioxygenated by potato LOX to a minor amount of 5(*S*),8(*R*)-dihydroperoxy-6(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [50]. In this case, O_2 placement was on the same side of the fatty chain, a behavior in agreement with the known 5(*S*)-, 8(*S*)-specificity of potato LOX. From all (*Z*)-8,11,14-eicosatrienoic acid, potato LOX furnished the 8(*S*),15(*R*,*S*)-dihydroperoxides; apparently, the less preferred oxygenation at carbon-15 was too far removed from the carboxylic acid signal to exert stereospecificity.

IV-B. Leukotrienes and lipoxins

It is remarkable that plant LOXs catalyze the production of bioactive compounds of mammalian origin, such as the leukotrienes and lipoxins. Ordinarily, these substances are not observed in the plant kingdom,

because the prevalent plant fatty acids do not have the requisite structures. The formation of leukotriene A and lipoxin A is dependent on the specificity of LOXs and their action on eicosanoids, rather than anything of animal origin. If one supplies arachidonic acid to a plant LOX, such as from potato [24,50,77], several leukotrienes and leukotriene-like isomers are produced from intermediate 5*S*-HPETE (Fig. 3a). Potato LOX apparently caused this transformation by virtue of its dual 5(*S*)- and 8(*S*)-LOX activity; that is, the 5*S*-HPETE was directly converted into the unstable 5,6-epoxide, leukotriene A_4, by abstraction of a pro-(*R*) hydrogen from the C-10 methylene by the 8(*S*)-LOX activity. Although leukotriene A_4 was too unstable to be demonstrated by ordinary methods, the epoxide solvolysis products, stereoisomeric 5,6-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic and 5,12-dihydroxy-6(*E*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acids were isolated and identified. The intermediacy of leukotriene A_4 was shown by methanol trapping to afford epimeric 5(*S*)-hydroxy-12-methoxy-6,8,10,14-eicosatetraenoic acid [77]. Also, dihydroxyeicosatetraenoic acids were formed from monohydroperoxides under anaerobic conditions, showing that the diol did not arise from a double dioxygenation step [50,78]. Similarly, potato LOX appeared to catalyze the formation of 8,9-leukotriene A_3 from all (*Z*)-8,11,14-eicosatrienoic acid based on the evidence that all (*E*)-8(*S*),15(*R*,*S*)-dihydroxy-9,11,13-eicosatrienoic acids are formed by incubation of potato LOX with the 8(*S*)-hydroperoxide [78]. Monohydroperoxides that are structural candidates for formation of leukotriene A caused inactivation of soy and potato LOXs, but evidence of inactivation by leukotriene A was circumstantial [79,80].

Lipoxins, another class of bioactive compounds produced by mammalian leukocytes, have been produced by the action of soy LOX, after $NaBH_4$ reduction of the product mixture [81]. As seen in Fig. 3b, the basic mechanism of hydrogen removal by LOX prior to epoxide formation was similar to leukotriene biosynthesis, except the precursor was a dihydroperoxide instead of a monohydroperoxide. Isomeric 5,6,15-trihydroxy-7,9,11,13-eicosatetraenoic and 5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acids were isolated only after $NaBH_4$ reduction of the product mixture, suggesting that the precursor was an epoxyhydroperoxyeicosatetraenoic acid. Either arachidonic acid or 5(*S*),15(*S*)-dihydroperoxy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid served as substrates for lipoxin formation.

IV-C. Products from anaerobic cycling

Garssen and co-workers reported in 1971 and 1972 that soy LOX catalyzed an anaerobic reaction with the substrates, linoleic acid and 13*S*-HPOD. It is now known that the reaction was caused by a ferric/ferrous redox cycle which generated pentadienyl radical from linoleic acid and alkoxyl radical from 13*S*-HPOD and indeed, known free radical reactions could explain the

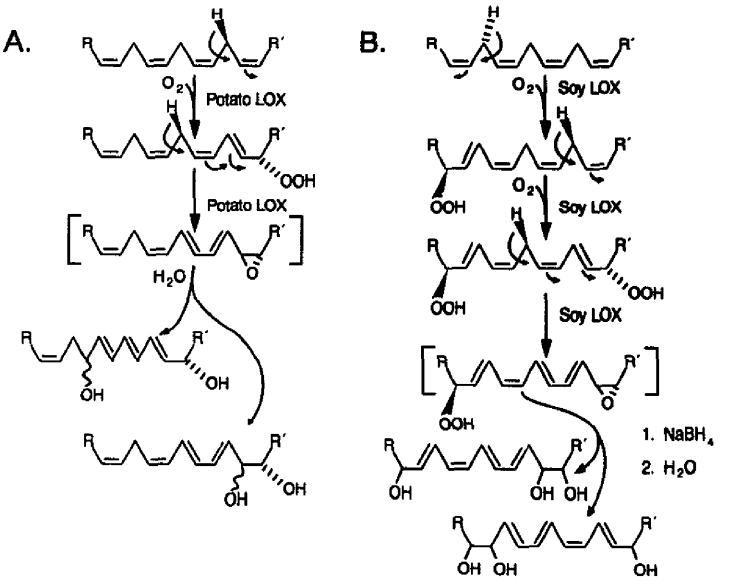


Fig. 3. (a) Conversion of arachidonic acid into leukotrienes by potato lipoxygenase [50,77]; (b) soybean lipoxygenase-catalyzed formation of lipoxins from arachidonic acid [81]. Stereospecific hydrogen removal and dioxygenation by the lipoxygenases are shown; R, $CH_3(CH_2)_4$- and R', $-(CH_2)_3COOH$.

products formed (Fig. 2). Some have described the anaerobic function of LOX as a 'hydroperoxidase cycle' [44], or as a leakage of radicals from the active site inversely dependent on the concentration of O_2 [66]. It is important to recognize that the normal aerobic cycle is in constant competition with the anaerobic cycle. Although O_2 supply may seem to be sufficient, a relatively high concentration of both substrate and enzyme may easily overwhelm the ability of the diffusion of O_2 to keep pace with the reaction. The appearance of ketodiene absorbance at 270–280 nm in LOX assays, due to the presence of 13-oxooctadecadienoic acid and 13-oxotridecadienoic acid, is diagnostic of insufficient oxygenation. For example, anaerobic-type products have been detected in aerobic incubations held at 37°C, instead of 20°C normally used [82], a result possibly due to 31% less O_2 solubility at 37°C compared to 20°C.

The three LOX isozymes of soybean differed in their requirement for O_2 in generating the anaerobic-type 270–280 nm absorbing compounds. LOX-1 required a more strict anaerobic environment; whereas, LOX-2 and in particular LOX-3, generated 270–280 nm absorption even in the presence of air [3]. And, the ability of these three isozymes to cause bleaching of either carotenoids or the dye, 2,6-dichlorophenolindophenol, correlated with their ability to produce 270–280 nm absorbing compounds [3]. The bleaching action is a cooxidation that some workers believe is caused by peroxyl radical. A possible origin of peroxyl radical could be from radical leakage from the enzyme as follows:

$$\text{LOX-Fe2+} - \text{R}^{\cdot} \longrightarrow \text{LOX-Fe2+} + \text{R}^{\cdot}$$

$$\text{R}^{\cdot} \xrightarrow[\text{O}_2\text{ (not strictly anaerobic)}]{} \text{ROO}^{\cdot}$$

$$\text{LOX-Fe3+} - \text{ROOH} \rightleftharpoons \text{LOX-Fe2+} - \text{ROO}^{\cdot}$$

$$\longrightarrow \text{LOX-Fe2+} + \text{ROO}^{\cdot}$$

Thus, the isozymes of soybean may differ by their propensity to leak radicals. The formation of singlet O_2 by LOX-3 in the presence of 13*S*-HPOD and linoleic acid was cited as an indication of the presence of peroxyl radical [83]. Peroxyl radical combination and subsequent rearrangement is known to produce singlet O_2 by the 'Russell Mechanism'. LOX-1 produced singlet O_2 only at very low O_2 concentrations in apparent agreement of its more strict requirement for anaerobic conditions. In contrast, only spin-trapped alkoxyl and acyl radicals, not peroxyl radicals, have been detected by EPR during an anaerobic reaction of LOX [84]; thus, there remains some uncertainty about the nature of the cooxidant. On the basis of differential effects of antioxidants on the bleaching of carotenoids and chlorophyll, it has been suggested that there are multiple sites for the anaerobic bleaching by soy LOX-1 and that the probable source of radicals are from both enzyme-bound and non-bound pentadienyl and alkoxyl radicals [85,86]. This proposal would seem to be more in agreement with the known fatty acid end-products and the EPR result.

Recent investigations of anaerobic products have focussed on those arising from arachidonic acid and its hydroperoxides. Products were analogous to those obtained previously with linoleic acid (Fig. 2). For example, anaerobic treatment of the 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (15*S*-HPETE) by soy LOX, in the absence of arachidonic acid, led to the formation of 13(*R*)-hydroxy-14(*S*),15(*S*)-epoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid [87]. However, with arachidonic acid included in the anaerobic incubation 15*S*-HPETE was converted into 15-oxo-eicosatetraenoic acid and 15-oxopentadecatetraenoic acid [82]. Similarly, incubation of 12(*S*)-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (12*S*-HPETE) and 8,11,14-eicosatrienoic acid afforded 12-oxoeicosatetraenoic acid and 12-oxododecatrienoic acid. Smaller yields of these latter products even formed aerobically indicating a more facile radical reaction using 12*S*-HPETE [82]. Interestingly, animal LOXs also produce these latter two compounds during anaerobic incubation of 12*S*-HPETE in the presence of substrate fatty acids [82,88]. A significant activation of leukocytes occurred in the presence of 12-oxododecatrienoic acid [88].

V. Hydroperoxide dehydrase

Hydroperoxide dehydrase (HPDS) is the revised nomenclature for an enzyme that was previously thought to be two separate enzymes, namely 'hydroperoxide isomerase' and 'hydroperoxide cyclase'. Research dates from the discovery of 'hydroperoxide isomerase' by Zimmerman in 1966 and 'hydroperoxide cyclase' by Zimmerman and Feng in 1978. Early work was completed in the 1970s and 80s mainly in the labs of Zimmerman and Vick, Veldink and Vliegenthart, Grossman and Gardner. Reviews are available that detail the early research [2,4,5,8,9,89,90], as well as recent work [91,92].

HPDS occurs in most cereal seeds or seedlings (reviewed in 9), flaxseed, lettuce, sunflower, spinach, cotton seedlings (reviewed in 8), *Vicia faba* [93], eggplant [94], *Chlorella pyrenoidosa* [95], possibly rat testis microsomes [96] and two species of coral [97,98]. The occurrence of HPDS in the plant and animal kingdoms affords yet another example of the ubiquitous nature of LOX pathway enzymes. The apparent lack of HPDS in certain plants is enigmatic; however, the enzyme may be either present at low levels or unstable during

TABLE I

Properties of hydroperoxide dehydrases recently characterized

Source	Size (kDa)	Substrate specificity [a]				pH optimum	Inhibitors	K_m (mM) (substrate)	References
		position of hydroperoxide		enantiomer					
		C-9	C-13	R	S				
Maize	> 130	+ [b]	+ [b]	n.d.	+ [b]	6.6 [b]	PCMB [c], CH_3HgI	$7 \cdot 10^{-2}$ [b] (HPOD)	94, 111
Eggplant	293	+	+	n.d.	+	6.5	$CuCl_2$, NDGA PCMB, NBS CH_3HgI, iodoacetate	$2.4 \cdot 10^{-2}$ (HPOD)	94
Spinach leaf	220	n.d.	+	n.d.	+	5–7			100
Cotton seedling	250	+	+ [d]	n.d.	+	5, 6.5			99
Flaxseed		+ [b]	+ [b,d]	–	+	7 [b]	$CuCl_2$ [b]		101

[a] n.d., not determined.

[b] Known from literature prior to 1980.

[c] PCMB, *p*-chloromercuribenzoate; NDGA, nordihydroguaiaretic acid; NBS, *N*-bromosuccinate.

[d] Isomer that is preferentially metabolized.

homogenization. For example, with *Chlorella pyrenoidosa* the presence of HPDS was only inferred by the presence of a product of the enzyme in homogenates [95]. HPDS is a membrane-bound enzyme that can be readily solubilized by detergents, such as Tween 20 [94] or Triton X-100 [99,100]. The enzyme is routinely isolated as a microsomal pellet, but the specific identity of the membrane association has been incompletely researched. In spinach leaves HPDS, as well as HPLS, are associated with whole and broken chloroplasts [100].

The properties of HPDS are summarized in Table I. Of particular interest is the specificity for substrate. Usually, both 9*S*-HPOD and 13*S*-HPOD served as substrates, but certain HPDSs had a preference for 13*S*-HPOD (e.g., Ref. 99). The specificity for stereoisomers is not fully known, but flaxseed metabolized only 13*S*-HPOT from the isomeric mixture, 13*R,S*-HPOT [101]. In contrast to the HPDSs of higher plants, the corresponding enzyme from the corals, *Plexaura homomalla* [98] and *Clavularia viridis* [97], metabolized

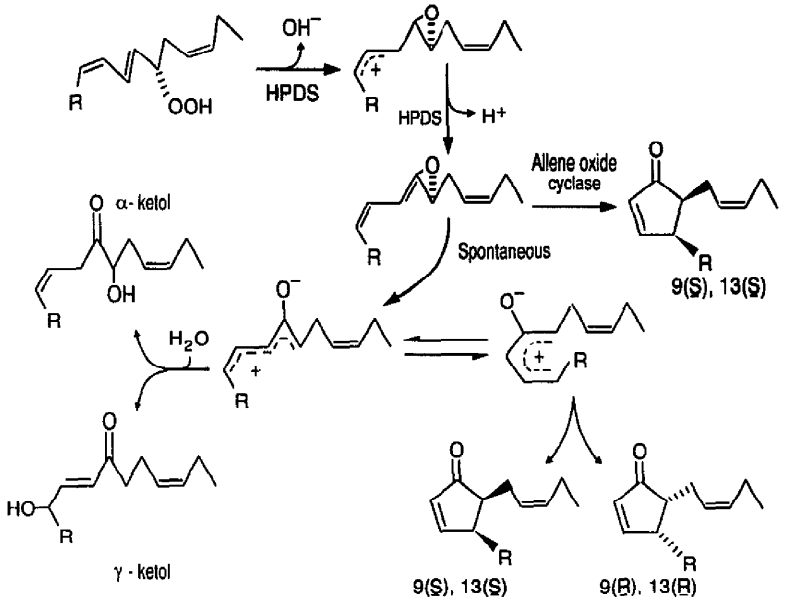


Fig. 4. Metabolism of 13*S*-HPOT by sequential action of hydroperoxide dehydrase (HPDS) and allene oxide cyclase, including spontaneous nonenzymic reactions of the HPDS product, allene oxide. Modified from [110] to show proposed cationic intermediates; R,-$(CH_2)_7$COOH.

8(*R*)-hydroperoxy-5(*Z*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid (8*R*-HPETE), which was of the opposite relative configuration compared to 9*S*-HPOD.

Formerly, the action of HPDS was attributed to two separate enzymes. Accordingly, 'hydroperoxide isomerase' was supposed to directly catalyze the transformation of HPOD and HPOT into α- and γ-ketols and 'hydroperoxide cyclase' supposedly transformed 13*S*-HPOT into 12-oxophytodienoic acid, 8-[2-(2'(*Z*)-pentenyl)-3-oxo-4(*Z*)-cyclopentenyl]octanoic acid. According to Gardner, as well as Vick and Zimmerman, a plausible pathway to the ketols and 12-oxophytodienoic acid would be through conversion of hydroperoxydiene moiety into an intermediate epoxyallylic cation. Although the epoxyallylic cation is still thought to be a viable transition state by recent workers [98,102,104, 105], loss of a proton from this cation leads to an unstable compound, the allene oxide, which can be isolated and characterized with certain precautions. In 1987, four separate groups, apparently working independently with flax seed [102], maize seed [106] and two species of coral [97,98], suggested that the intermediate to formation of α- and γ-ketols, as well as 12-oxophytodienoic acid and its homologs was an allene oxide (Fig. 4). The probable existence of an allene oxide was also demonstrated by a biomimetic synthesis of 12-oxophytodienoic acid analogs [107].

It was Hamberg [106], who first demonstrated the presence of the allene oxide, 12,13(*S*)-epoxy-9,11-octadecadienoic acid (12,13*S*-EOD), as the progenitor of the α-ketol, 13-hydroxy-12-oxo-9(*Z*)-octadecenoic acid. The half-life of 12,13*S*-EOD was only about 33 s at 0°C. However, the methyl esters of the allene oxides were relatively stable in cold aprotic solvent, permitting Brash and co-workers [108] to isolate them after enzymic conversion by flaxseed HPDS and then characterize them by spectral methods as 12,13*S*-EOD methyl ester (from 13*S*-HPOD) and methyl 12,13-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoate (from 13*S*-HPOT). Similarly, an allene oxide from action of coral HPDS on 8*R*-HPETE has been isolated and characterized [109].

After the allene oxide is formed from 9*S*-HPOD or 13*S*-HPOD, spontaneous non-enzymic hydrolysis affords α- and γ-ketols (Fig. 4). Possibly because of the partial S_N2 character of OH^- substitution at the carbon originally bearing the hydroperoxide group, the hydroxyl group of the α-ketol was about 70% inverted to (*R*)-stereochemistry [106,108]. Similarly, the allene oxide from action of coral HPDS on 8*R*-HPETE hydrolyzed into an α-ketol with 70% inverted (*S*)-stereochemistry [98]. The γ-ketol was racemic undoubtedly due to S_N1 substitution on the delocalized carbocation proposed as the intermediate to solvolysis [110]. Because the configuration of the allene oxide is not fully known (the *cis* versus *trans* orientation of the epoxide and *Z* versus *E* configuration of the 11-double bond in the case of the 12,13-epoxyoctadecatrienoic acid), a conformational dilemma arises concerning the orientation of the carbocation intermediates shown in the center of Fig. 4. The equilibrium shown is simply an attempt to explain the observed stereoconfiguration of the products obtained.

The allene oxide from 13*S*-HPOT spontaneously converted into not only the ketols, but also into 12-oxophytodienoic acid at a level about 10% of total products. The spontaneously formed 12-oxophytodienoic acid was racemic [9(*S*),13(*S*) and 9(*R*),13(*R*)] with the alkyl chains in a *cis* configuration [101,108, 110,111]; the analogous cyclic fatty acid from action of coral HPDS on 8*R*-HPETE was also racemic with *cis* alkyl chains [98]. Since cyclization required 13*S*-HPOT as a substrate, but not 13*S*-HPOD, the cyclization of the allene oxide appeared to be dependent on the 15(*Z*) double bond. A similar relative position of the double bond existed in the allene oxide derived from the 9-hydroperoxide of γ-linolenic acid and a preliminary report has confirmed the spontaneous formation of the corresponding cyclic fatty acid [110]. A slight degree of cyclization occurred with 12,13*S*-EOD by flaxseed HPDS affording a trace of the corresponding 12-oxophytoenoic acid, 8-[3-oxo-2-pentyl-4(*Z*)-cyclopentenyl]octanoic acid [108]. Evidently, the presence of a double bond β to the allene oxide is not an absolute prerequisite.

Numerous labeling experiments confirmed the mechanistic details of formation and the intermediacy of the allene oxide. Incubation of either [9,10,12,13-$^2H_4$]linoleic acid or the identically labeled 13*S*-HPOD with flaxseed or maize preparations led to loss of ^2H at carbon-12 from the products, α-ketol [102,106] and γ-ketol [105], indicating loss of a proton to H_2O during formation of 12,13*S*-EOD. Incubation of substrate with HPDS in $H_2^{18}O$ or ^2H_2O resulted in incorporation of ^{18}OH [98] or one ^2H-label, respectively, in the α-ketol product [98,101]; however, the ^2H-labeling was somewhat confused by a small exchange with H_2O [98,101,102]. The $^{18}O_2$-labeled 13*S*-HPOD or 13*S*-HPOT furnished α-ketol or 12-oxophytodienoic acid, respectively, with retention of one ^{18}O at carbon-12 [106,108], a fact already known from earlier work. There was significant exchange of ^{18}O from the α-ketol, presumably by H_2O [108]. [14-$^2H_2$]linolenic acid afforded 12-oxophytodienoic acid with no loss of label as expected [104].

When the substrate, 8(*S*)-hydroxy-15(*S*)-hydroperoxyeicosapentaenoic acid, was incubated with the flaxseed HPDS, four isomeric prostaglandin A_3 (PGA_3) analogs, which differed in the configurations of the side chains (two with *cis* and two with *trans* alkyl chains), were formed in addition to two α-ketol isomers, 8(*S*),15-dihydroxy-14-oxo-5(*Z*),9(*E*),11(*Z*),

17(*Z*)-eicosatetraenoic acid and the corresponding 11(*E*) isomer [103]. The 11(*E*) α-ketol indicated the presence of an 11(*E*) allene oxide precursor in addition to the predicted 11(*Z*) isomer. This 11(*E*) allene oxide was proposed to account for the two PGA_3 analogs that had their alkyl chains in a *trans* configuration instead of the customary *cis*. Undoubtedly, the motivation behind the above study stemmed from the fact that the coral, *Plexaura homomalla*, contains 2–3% of its dry weight as PGA_2 methyl ester 15-acetate and also contains an active HPDS. Although the 8*R*-HPETE served as a substrate for *P. homomalla* HPDS in the formation of the nonhydroxylated prostanoid, 'pre-clavulone A' [97,98], various isomers of 8(*R*)-hydroperoxy-15(*R,S*)-hydro(pero)xyeicosatetraenoic could not be transformed into PGA_2 by coral HPDS [103]. Recently, workers have been examining minor metabolites of coral HPDS to possibly gain insight into PGA_3 biosynthesis. One such effort has afforded characterization of a new cyclopropyl eicosanoid product arising from an eicosanoid allene oxide [112].

According to Hamberg et al. [113], maize HPDS converted 13*S*-HPOT into 12-oxophytodienoic acid with a partial chiral purity; that is, an 82:18 ratio of 9(*S*),13(*S*):9(*R*),13(*R*) stereoconfiguration of the alkyl side chains. Subsequently, Hamberg's laboratory [110,111] found an allene oxide cyclase in maize, as well as several other plants, that specifically catalyzed the formation of the 9(*S*),13(*S*) enantiomer. This enzyme was a soluble protein with a molecular weight of about 45 000 found in the $105\,000 \times g$ supernatant. Thus, HPDS and allene oxide cyclase activities could be separated by centrifugation into $105\,000 \times g$ pellet and supernatant, as well as by gel filtration and ion exchange chromatography. By increasing the activity of allene oxide cyclase relative to HPDS and lowering the substrate concentration, the 9(*S*),13(*S*) chiral purity was increased to 98% and the percentage of 12-oxophytodienoic acid increased considerably at the expense of the spontaneous solvolysis products, the ketols [110,111]. A substrate mimic, ±*cis*-12,13-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, was found to be a potent inhibitor of the allene oxide cyclase [110].

In addition to H_2O, other protic substances react with the allene oxide, by nucleophilic displacement at the epoxide carbon. Christianson and Gardner reported in 1975 that methanol, ethanethiol, or oleic acid, if included in the reaction of 13*S*-HPOD catalyzed by HPDS, resulted in substitution of the anion at the carbon originally bearing the hydroperoxide group. At that time it was thought that the substitution was an integral part of the enzymic process, rather than nucleophilic attack on the allene oxide. By reacting hydroperoxide substrate with a large excess of HPDS at 0°C, a quickly formed allene oxide can be quenched in various reagents for study of allene oxide chemistry. For example, methanol trapping of the 12,13*S*-EOD had been utilized as a method to approximate its half-life [106]. Subjecting 12,13*S*-EOD to the aprotic solvent, acetonitrile, not only increased its half-life (7.4 min at 0°C in 95% acetonitrile), but also resulted in the formation of two isomeric macrolactones, 12-oxo-9(*Z*)-octadecen-11-olide and 12-oxo-9(*Z*)-octadecen-13-olide [114]. Even though the acetonitrile contained 5–10% H_2O, there was little hydrolysis into ketols. Rather, the preferred reaction was intramolecular attack on the allene oxide by the carboxyl group affording the macrolactones. As discussed previously, 12,13*S*-EOD furnished only trace amounts of the 12-oxophytoenoic acid from spontaneous cyclization, presumably due to the lack of a 15(*Z*) double bond [108]. However, in the presence of serum albumin 12-oxophytoenoic acid did form from 12,13*S*-EOD at a maximum level of 24% of recovered products [115]. Rather than the usual *cis* configuration of the alkyl side chains, *trans* and virtually racemic products [9(*R*),13(*S*); 9(*S*),13(*R*)] were formed. Bovine serum albumin was the most efficient of all the serum albumins tested in effecting this transformation and it was also the most effective in increasing the half-life of 12,13*S*-EOD (14 min at 0°C with 15 mg/ml bovine serum albumin).

VI. Hydroperoxide lyase

A membrane-bound HPLS, found in higher plants, catalyzes the cleavage of the fatty acid chain between the hydroperoxide group and the α-olefinic carbon, affording an aldehyde and an ω-oxoacid. Since this reaction resembles a known acid-catalyzed cleavage of the hydroperoxide, this type of enzyme is tentatively categorized here as a 'heterolytic' HPLS. On the other hand, a soluble enzyme from mushroom, two species of algae and one species of higher plants cleaves the fatty chain on the opposite side of the hydroperoxide-bearing carbon; that is, between the hydroperoxide carbon and the α-methylene resulting in an alcohol (or hydrocarbon) and an ω-oxoacid. Since this fragmentation resembles the β-scission of alkoxyl radicals derived from hydroperoxides, this HPLS is designated a 'homolytic' type. As a caveat, it cannot be assumed that either of these transformations is mechanistically defined, but these classifications are offered to distinguish the two.

VI-A. 'Heterolytic' hydroperoxide lyase

There was considerable research activity concerning the 'heterolytic' HPLS during the 1970s and specific reviews detail earlier work [116–119], including those reviews covering additional aspects of the LOX pathway [4,5,8,9,120].

TABLE II

Properties of the 'heterolytic'-type hydroperoxide lyase recently characterized

Source	Isozyme number	Substrate specificity [a]				pH optimum	Inhibitors [b]	K_m, (mM) (substrate)	Size (kDa)	References
		position of hydroperoxide		enantiomer						
		C-9	C-13	*R*	*S*					
Tomato		n.d.	+	n.d.	+	5.5	PCMB	$2.6 \cdot 10^{-3}$ (13*S*-HPOD)	200	122
Pea		+	–	n.d.	+	6.5		–		123
Soybean		– [c]	+	–	+	6–7		$4\text{-}6 \cdot 10^{-2}$ (13*S*-HPOD) 0.16 (13*S*-HPOT)	240–260	121, 126
Cucumber										
seedling	1	–	+	n.d.	+	8	PCMB			124
cotyledon	2	+	–	n.d.	+	6.5	IA, PCMB, PMSF	–		
Tea leaf										
chloroplasts		–	+	–	+	7–8	MB, DCPIP, SKF 525-A	1.9 (13*S*-HPOD) 2.5 (13*S*-HPOT)		128, 132
Spinach leaf										
chloroplasts		n.d.	+	n.d.	+	7–9		–	220	100

[a] n.d., not determined.

[b] PCMB, *p*-chloromercuribenzoate; IA, iodoacetate; PMSF, phenylmethanesulfonylfluoride; MB, methylene blue; DCPIP, 2,6-dichlorophenol indophenol; SKF 525-A, 2-dimethylaminoethyl-2,2-diphenyl valerate.

[c] This result conflicts with our unpublished work.

This type of HPLS is a membrane-bound enzyme requiring detergent solubilization (usually Triton X-100), which is presumably the reason for slow progress with its isolation. Most recently, several investigators purified detergent-solubilized HPLS by chromatography [100,121–124] and estimated its size at 200–260 kDa (Table II). Table II also summarizes the properties of several HPLSs.

HPLS is expressed at various levels in tissues or organelles of higher plants. For example, in maize seeds HPLS appeared to be absent due to the predominance of HPDS, but HPLS activity was found in maize leaves [125]. On the other hand, HPLS was found in both the seeds [121,126] and leaves [125] of soybeans. The root possessed the highest activity in cucumber seedlings [127]. In leaves the enzyme was mainly localized in chloroplast membranes (100,128, reviewed in Ref. 116), which presumably led to a study that correlated HPLS activity from the leaves of various plants with their chlorophyll content [125]. In fruit tissue of

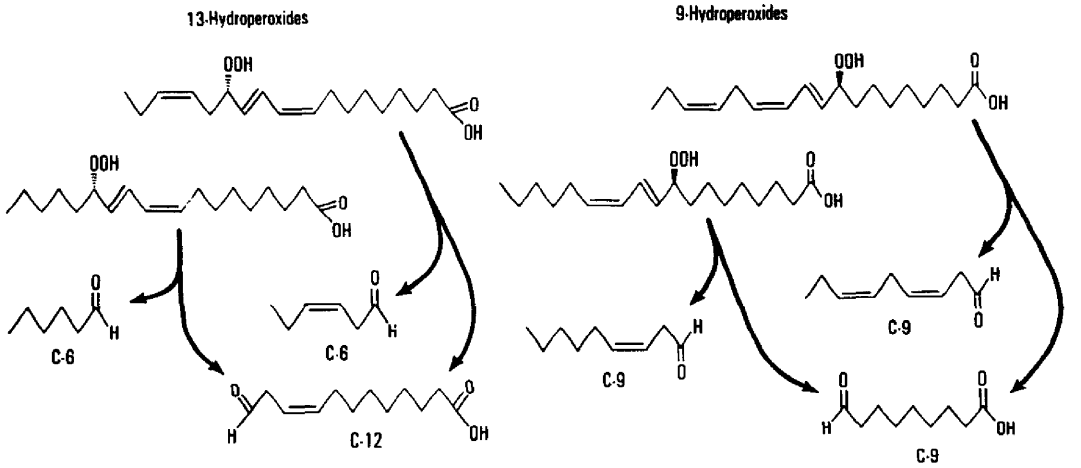


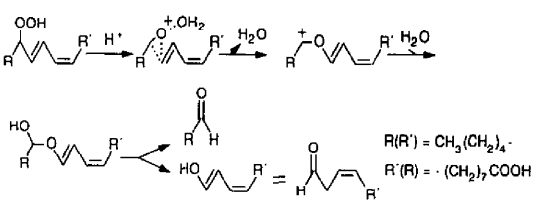
Fig 5. 'Heterolytic'-type hydroperoxide lyase activity with hydroperoxides of linoleic and linolenic acids showing either C-6/C-12 or C-9/C-9 fragmentations. Reprinted from Ref. 119.

cucumber, HPLS appeared to be localized in Golgi, plasma and endoplasmic reticulum membranes (reviewed in Ref. 117). Levels of activity reportedly also changed with exposure of plants to different environmental conditions [129–131].

HPLS affords cleavage of either 9-hydroperoxides or 13-hydroperoxides into either two C-9 or a C-6 and C-12 fragment, respectively (Fig. 5). By the 1970s it was established that different plant tissues had unique specificities for substrate hydroperoxide; thus, the existence of isozymes was implied (Table II). For example, pear fruit HPLS was specific for 9*S*-HPOD and 9*S*-HPOT, but not the corresponding 13-hydroperoxides [123]. Both tomato and cucumber HPLS were active with 13*S*-HPOD and 13*S*-HPOT, but only cucumber also utilized 9-hydroperoxides (reviewed in Ref. 119). From cucumber seedlings the activities specific for 9*S*-HPOD or 13*S*-HPOD were separated by chromatography showing that the two activities were actually due to isozymes [124].

The substrate (*S*)-stereospecificity of HPLS is implied since LOX-produced hydroperoxides are commonly used to study the enzyme. Recently, it has been shown that the 13*R*-HPOD did not serve as a substrate for soybean seed HPLS, nor were either of the (*E*),(*E*)-diene isomers of 13*R*,*S*-HPOD used by the enzyme [126]. Although soybean seed/seedling HPLS has been reported to be inactive with 9*S*-HPOD [121,126], we have observed the specific utilization of the 9(*S*) enantiomer from a 9*R*,*S*-HPOD mixture by soybean seed HPLS (Gardner et al., unpublished). HPLS from tea leaf chloroplasts showed a preference in metabolizing the 13*S*-HPOT enantiomer from a 13*R*,*S*-HPOT mixture [132]. With tea leaf chloroplast HPLS, the 13*S*-HPOT and 13*S*-HPOD substrates were preferred over the soy LOX-generated hydroperoxides of γ-linolenic acid, methyl linoleate and linoleoyl alcohol [128].

As shown in the following scheme, it may not be coincidental that the acid-catalyzed Hock-Criegee Rearrangement of either 13*S*-HPOD or 9*S*-HPOD afforded the same products as those catalyzed by HPLS [133]:



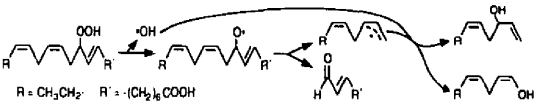
Experimental support for this mechanism has been demonstrated by the use of $^{18}O_2$-labeled 13*S*-HPOD substrate. First, there was a significant ^{18}O-isotope effect and second, the ^{18}O-label was transferred to 12-oxo-9(*Z*)-dodecenoic acid, but not to hexanal [134,135] in accord with the scheme above.

As might be expected, the aldehyde products of HPLS have odors commonly associated with the plants that produce them. Thus, the odors of hexanal or 3(*Z*)-hexenal have been described as green/rancid/beany or green/grassy, respectively. The odors of 3(*Z*)-nonenal and 3(*Z*),6(*Z*)-nonadienal have been related to those associated with cucumbers, pears and violets. The flavor profile of the aldehydes are further modified by the subsequent isomerization of the 3(*Z*)- into 2(*E*)-enals by enzymic and nonenzymic processes (reviewed in Ref. 117). For example, the odor of 2(*E*)-hexenal is a fresh spicy-green in contrast to the intense green-grassy note of 3(*Z*)-hexenal. The nonvolatile C-12 fragment, 12-oxo-9(*Z*)-dodecenoic acid, also isomerizes into the corresponding 10(*E*) isomer [117,136]. And, the various aldehydes are susceptible to conversion into alcohols by alcohol dehydrogenase causing further flavor modification [137].

It has been reported [138] that fish possess HPLS activity, possibly explaining why fresh fish have a green-cucumber odor. Once again this observation gives yet another example of crossover between the plant and animal kingdoms in enzymes of the LOX pathway.

VI-B. 'Homolytic' hydroperoxide lyase

The so-called 'homolytic' HPLS pathway is illustrated in Fig. 6. This activity is similar to the anaerobic reaction of LOX, which leads to the formation of alkoxyl radicals and β-scission to similar fragments; however, the resemblance is superficial. First, this HPLS was nonidentical to and separable from LOX [95,139]. Secondly, in mushroom extracts the enzyme was stereospecific for the 10-(S)-enantiomer, 10(*S*)-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid (10*S*-HPOD) and the alcohol product was only the (*R*)-enantiomer [140]. The stereospecificity for the 10-hydroperoxy-8(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (10-HPOT) substrate was not directly determined; however, one of the three products, 1,5-octadien-3-ol was also the *R*-enantiomer [141]. Although a concerted mechanism was proposed [140], the formation of products could just as easily be explained chemically by a homolytic route:



Apparently, both oxygens were transferred from $^{18}O_2$-labeled 10*S*-HPOD into each of the two cleavage fragments, 1-octen-3-ol and 10-oxo-8(*E*)-decenoic acid, but the results were not unambiguous because of partial exchange of the latter product with unlabeled H_2O [142].

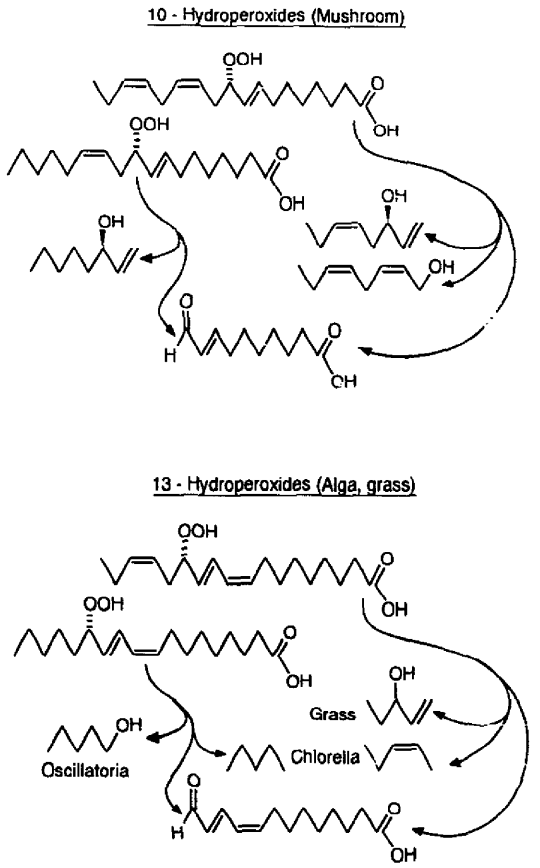


Fig. 6. Reactions of the 'homolytic'-type hydroperoxide lyase arising from either 10(*S*)-hydroperoxides or 13(*S*)-hydroperoxides of linoleic and linolenic acids. The pathways shown occur in mushroom [140,141], *Oscillatoria* sp. [139], the grass *Agropyron repens* [144] and *Chlorella pyrenoidosa* [95]. The (*S*)-stereospecificity of 10*S*-HPOT is implied, but not directly demonstrated, from the known (*S*)-specificity for 10*S*-HPOD [140].

At this time, the 'homolytic'-type HPLS has been mainly found in the lower plant forms, the fungi [143] and algae [95,139]. It has been reported that the grass, *Agropyron repens*, formed volatile products characteristic of both types of HPLS [144]. Although the presence of LOX in the *A. repens* preparation obscured the unambiguous identification of a homolytic-type HPLS, the determination of the cleavage fragment as 1-penten-3-ol categorized the reaction as being more similar to those catalyzed by the HPLSs of *Oscillatoria* [139] and mushroom [143], than of an anaerobic reaction of LOX.

Other features of this type of HPLS are furnished in Table III. It is of special interest that this class of enzyme is smaller in size than both LOX and the heterolytic-type HPLS. It can also be seen in Table III that the mushroom HPLS required the most exotic substrates, 10*S*-HPOD and 10-HPOT, implying the presence of a 10(*S*)-specific LOX. Such a LOX was thought to be present in mushroom, but was too unstable to be characterized [143].

VII. Other hydroperoxide activities

Several other enzymic systems have been identified that are active with substrate fatty acid hydroperoxides. These enzymes appear to be less widely distributed among the plant kingdom.

VII-A. Divinyl ethers

During the early 1970s, Galliard and co-workers reported that a potato extract catalyzed the transformation of either 9*S*-HPOD or 9*S*-HPOT into the divinyl ether fatty acids, 9-[1′(*E*),3′(*Z*)-nonadienyloxy]8-(*E*)-nonenoic acid or 9-[1′(*E*),3′(*Z*),6′(*Z*)-nonatrienyloxy]8(*E*)-nonenoic acid, respectively (reviewed in Ref. 5). These compounds were named colneleic and colnelenic acids after Colney Lane, the street address of Galliard's research institute. Labeling experiments have been utilized to probe the enzyme's mode of action. The ^{18}O-label from $^{18}O_2$-labeled 9*S*-HPOD was inserted into the ether oxygen of the divinyl ether [145] and incubation of [9,10,12,13-$^2H_4$]-9*S*-HPOD with the potato enzyme resulted in retention of all four deuteriums [102]. In addition, there was a stereospecific re-

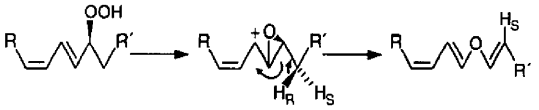
TABLE III

Properties of the 'homolytic'-type HPLS

Source	Substrate specificity [a]					pH optimum	Inhibitors	K_m (mM)	Size (kDa)	References
	position of hydroperoxide			enantiomer						
	C-9	C-10	C-13	*R*	*S*					
Chlorella pyrenoidosa	n.d.	n.d.	+	n.d.	+	6–8.3			48	95
Oscillatoria sp.	–	n.d.	+	n.d.	+	6.4	quercetin	0.0074	56	139
Mushroom	–	+	–	–	+	6.5				140–143

[a] n.d., not determined.

moval of the pro-(*R*) hydrogen at C-8 [146]. These results suggested a proposed heterolytic rearrangement [145,146], which was very similar to that proposed above for HPDS, as well as the heterolytic HPLS [146]:

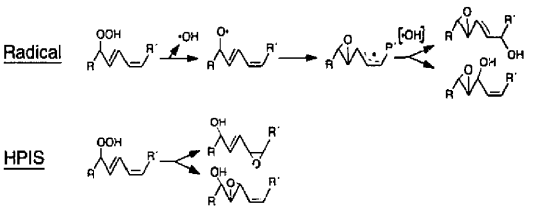


R = $CH_3(CH_2)_4$-; R' = -$(CH_2)_6COOH$

Knowledge of the mechanism has been used to synthesize divinyl ether from 9*S*-HPOD by a biomimetic method [71].

VII-B. Hydroperoxide isomerase

Hydroperoxide isomerase (HPIS) converts fatty acids into epoxyhydroxy fatty acids. This enzyme is totally different from the 'hydroperoxide isomerase' that was recently renamed HPDS. During the 1970s numerous reports appeared of various HPISs in cereal flours. Unfortunately, the structural data on the products, for the most part, were too ambiguous to assign a mechanism (reviewed in Ref. 9); that is, it could not be determined if the activity was due to an HPIS, as described herein, or a rearrangement of the alkoxyl radical, similar to that catalyzed by the anaerobic reaction of LOX. There is a fundamental difference in the structures of the products when an alkoxyl radical reaction is compared to an HPIS catalyzed process as follows:



Recently, a genuine HPIS from the fungus, *Saprolegnia parasitica*, has been characterized [147–151]. Hamberg [147] reported the transformation of arachidonic acid into isomeric trihydroxyeicosatrienoic acids by cultures of the fungus. Subsequent work with homogenates of the fungus showed that the trihydroxyeicosatrienoic acids originated from a sequence starting with LOX oxidation of arachidonic acid into 15*S*-HPETE, rearrangement of the hydroperoxide into two isomeric epoxyhydroxyeicosatrienoic acids [148] and the subsequent hydrolysis of the epoxide group affording four isomeric trihydroxyeicosatrienoic acids [149]. Specifically, 15*S*-HPETE was converted by HPIS into 11(*S*),12(*R*)-epoxy-15(*S*)-hydroxy-5(*Z*),8(*Z*),-13(*E*)-eicosatrienoic acid and 13(*R*),14(*R*)-epoxy-15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid [149]. Since

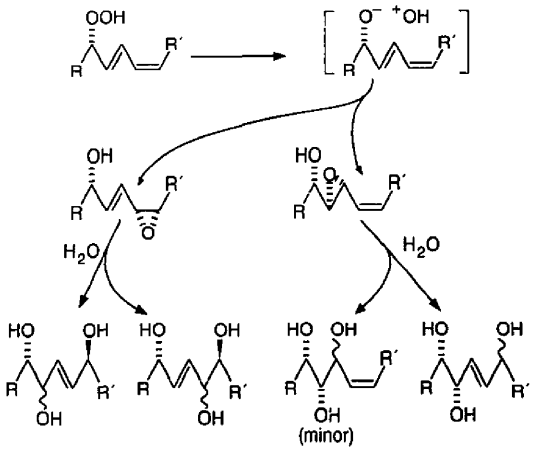


Fig. 7. Conversion of linoleic acid 9(*S*)- or 13(*S*)-hydroperoxides into epoxyhydroxyoctadecenoic acids by hydroperoxide isomerase and the subsequent hydrolysis of the epoxides. R, $CH_3(CH_2)_4$- and R'-$(CH_2)_7COOH$; or R-$(CH_2)_7COOH$ and R', $CH_3(CH_2)_4$-. The pathway was constructed from the studies of Hamberg et al. [148,151,152].

each of the two oxygens from the hydroperoxide group was transferred intramolecularly to the epoxide and the hydroxyl groups, this identified the enzyme as a true isomerase [148]. In addition to utilizing 15*S*-HPETE as a substrate, 13*S*-HPOD and 9*S*-HPOD also were metabolized by the fungal HPIS furnishing analogous products with the same relative regio- and stereo-chemistry of the epoxy and hydroxy groups (Fig. 7), as compared to products of 15*S*-HPETE [151]. The pathway of hydrolysis of the epoxyhydroxyoctadecenoic acids was also defined by Hamberg [151], Fig. 7. The other enantiomers, 13*R*-HPOD and 9*R*-HPOD, were poorly metabolized by HPIS [151]. A biomimetic route to 11,12-epoxy-13-hydroxy-9(*Z*)-octadecenoic acid from 13*S*-HPOD was recently shown to be possible with a vanadium catalyst [152].

Although a major portion of the fungal HPIS sediments at $105\,000 \times g$ [151], activity was also noted in the soluble fraction [150]. And, the LOX activity of *S. parasitica* could not be separated from the soluble HPIS activity using three different chromatographic systems [150]. Moreover, both activities were similarly affected by pH, inhibitors and temperatures indicating that LOX and HPIS activities resided on the same protein or protein complex. The size of the protein was about 145–150 kDa. This poses an interesting theoretical question about the enzyme mechanism, because the anaerobic reaction of LOX usually catalyzes a rearrangement of alkoxyl radical which is clearly different from the type of reaction catalyzed by this LOX/HPIS activity [148].

VII-C. Hydroperoxide-dependent peroxygenase and hydroperoxide-dependent epoxygenase

Numerous workers have reported the formation of a small percentage of hydroxydiene or hydroxytriene fatty acids as a consequence of exposing the corresponding hydroperoxides of linoleic or linolenic acid to plant extracts; however, few have examined this conversion in detail. One such route to hydroxy fatty acids may be via a microsomal hydroperoxide-dependent peroxygenase (HPPR) or hydroperoxide-dependent epoxygenase (HPEP). As will be discussed below, HPPR and HPEP have some common features in their ability to both transform hydroperoxide fatty acids into the corresponding hydroxy fatty acids and cooxidize unsaturated fatty acids into their corresponding epoxide fatty acids. Since these two enzymes afford differences in the stereospecificity of epoxidation, it is prudent at this time to retain the separate nomenclatures suggested by the various investigators.

In 1977 Ishimaru and Yamazaki reported that a peroxygenase (a hemoprotein), from pea microsomes catalyzed the hydroxylation of a substrate, like phenol, aniline, indole, or 1-naphthol, by utilizing linoleic acid hydroperoxide as the oxidant. As a result, the hydroperoxide was transformed into the corresponding alcohol. Soybean microsomes also contained a HPPR and a novel oxidation of sulfides to sulfoxides has been reported recently [153,154]. Thus, the pesticide, methiocarb and methyl *p*-tolyl sulfide were converted to their sulfoxides with a corresponding conversion of fatty acid hydroperoxide into its alcohol. By use of $^{18}O_2$-labelled 13*S*-HPOD it was determined that the sulfoxide oxygen originated from hydroperoxide [153]. And, methyl *p*-tolyl sulfide was stereospecifically oxidized to primarily the (*S*)-sulfoxide [154].

Microsomal HPPR from soybean [155] and microsomal HPEP from *Vicia faba* [156] both catalyzed the epoxidation of unsaturated fatty acids in the presence of 13*S*-HPOD. Like the reactions described above, there was a mole to mole epoxidation of olefin to hydroperoxide reduced and the epoxide acquired one ^{18}O-label from $^{18}O_2$-labeled 13*S*-HPOD. Moreover, the soybean epoxygenase and sulfoxygenase activities coeluted by CM-Sepharose chromatography indicating that the same HPPR was involved [155]. Both soybean HPPR and *V. faba* HPEP converted oleic acid into its 9,10-epoxide and linoleic acid afforded 9,10- and 12,13-epoxides [155,156]. Additionally, soybean HPPR could transform linoleic acid into the 9,10,12,13-diepoxide [155]. As expected the epoxides retained the *cis* configuration of the substrate (*Z*)-unsaturated fatty acids; however, there also was a preference for formation of one of two chiral pairs of epoxides [156,157]. With *V. faba* HPEP the 9(*R*),10(*S*) and 12(*S*),13(*R*) enantiomers predominated over 9(*S*),10(*R*) and 12(*R*),13(*S*) [156], but with soybean HPPR there was an enantiomeric excess of 9(*R*),10(*S*) and 12(*R*),13(*S*) over 9(*S*),10(*R*) and 12(*S*),13(*R*) [157]. That is, the 9,10-epoxides formed by the two enzymes have the same configuration, but the 12,13-epoxides have the opposite stereochemistry.

The *V. faba* HPEP also afforded an additional epoxide fatty acid. The reduced hydroperoxide, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid became epoxidized into 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid and a minor amount of its enantiomeric 9(*R*),10(*S*)-epoxide [156]. Although the predominant enantiomer of this isomeric pair was identical to a product of HPIS described above (Fig. 7), the mechanism of its formation was decidedly different. By use of mixtures of $^{16}O_2$- and $^{18}O_2$-labeled 13*S*-HPOD, it was shown that O-transfer to epoxide occurred by both inter- and intramolecular transfer from hydroperoxide [156] in contrast to the strict intramolecular O-transfer by HPIS.

The role of HPPR or HPEP is not known in the observed formation of hydroxy fatty acids by drying leaves. Large quantities of hydroxytriene and oxotriene fatty acids, apparently originating from LOX oxidation of linolenic acid, accumulated during the drying of leaves. Thus, 9(*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid and lesser amounts of its corresponding oxotriene fatty acid occurred in amounts equivalent to one eighth of the linolenic acid residues found in the dried leaf of *Glechoma hederacea* [158].

VIII. Physiological significance

When one considers the number of LOX isozymes in plants, their localization in specific tissues [159,160] and the variety of enzymes that metabolize the product hydroperoxides of LOX, it would seem that a role in plant physiology would be assured. Unlike research of the physiological effects of the LOX pathway in animals, corresponding progress in plant research has been somewhat disappointing with the notable exception of the origin of the plant hormone, jasmonic acid, from a HPDS product. The role of LOX in phytoalexin formation, abscisic acid biosynthesis and ethylene generation from precursor 1-aminocyclopropane-1-carboxylic acid has been suggested, but unsubstantiated. Others have suggested a role for LOX in plant senescence [161] and the control of metabolic pathways by LOX oxidation of sulfhydryl enzymes [162]; that is, LOX could act as an antagonist of thioredoxin. Those areas of research that are currently more experimentally convincing are reviewed here.

VIII-A. Jasmonic acid

The product of HPDS, 12-oxophytodienoic acid, was metabolized by *Vicia faba* [93] and a number of other

plant species [163] into the plant hormone, jasmonic acid. The metabolic steps included a reductase that saturated the cyclopentenone ring [164] followed by cleavage of six carbons from the carboxylic acid portion of the molecule by three β-oxidation steps. Inasmuch as 12-oxophytodienoic acid with 9(*S*),13(*S*) alkyl side chains probably is the true metabolic precursor of this pathway [110], (+)-7-iso-jasmonic acid would be the end-product. In this regard, it is interesting that (+)-7-iso-jasmonic acid had higher biological activity in the growth inhibition of wheat and rice seedlings than (−)-jasmonic acid [165]. It was also found that there was spontaneous isomerization of (+)-7-iso-jasmonic acid into (−)-jasmonic acid.

Jasmonic acid and/or its methyl ester has often been cited to cause growth inhibition, abscission, senescence, induction of certain leaf proteins, as well as other effects. Among the most exciting developments in the recent literature is a report that methyl jasmonate, when applied to tomato plants, induced the biosynthesis of defensive proteinase-inhibitor-proteins in the leaves at higher levels than can be obtained by wounding [166]. Moreover, a similar response was obtained when the plants were exposed to vapor phase methyl jasmonate in enclosed chambers. The hormone was applied either by direct vaporization or by proximity to a plant, *Artemisia tridentata*, known to contain methyl jasmonate in its leaves. These observations imply that methyl jasmonate may furnish plants with a mechanism for remote signalling.

VIII-B. Wounding and disease resistance

A large increase in LOX activity was induced by wounding of soybean leaves, either mechanically or by feeding of the spider mite [167]. The increase in activity was due to increases in both protein and transcripts. Also, wounding activated the HPLS pathway to give 12-oxo-10(*E*)-dodecenoic acid, which reportedly has wound healing properties [168].

Certain LOX pathway products have antifungal properties. Rice plants infected with rice blast fungus, *Pyricularia oryzae*, produced self-defensive substances, which were attributed to LOX products of linoleic and linolenic acid. Several isomeric hydroxydiene and hydroxytriene fatty acids of probable LOX origin were isolated [169,170], as well as isomeric trihydroxyene, trihydroxydiene and epoxyhydroxydiene fatty acids [171–173]. These latter compounds were structurally related to those produced by the rearrangement of alkoxyl radicals derived from 13-hydroperoxides of linoleic and linolenic acids. Five different isomeric *cis*-epoxides of linoleic and linolenic acids were also determined to possess anti-fungal activity against rice blast disease [170]. It is conceivable that these epoxides were formed by action of enzymes similar to HPPR or HPEP described above. One of the epoxides was fully characterized stereochemically as 12(*R*),13(*S*)-epoxy-9(*Z*)-octadecenoic acid [170], which is structurally identical to one of the products of soybean HPPR action on linoleic acid [157]. Also, the trihydroxyene fatty acid, 9,12,13-trihydroxy-10(*E*)-octadecenoic acid, was found to be produced by taro tubers as a defensive substance against the black rot fungus, *Ceratocytis fimbriata* [174]. Several workers have noted the antifungal properties of the HPLS products, hexanal, hexenal and nonenal (Ref. 175 and Refs. therein). However, it is this author's opinion that many other, possibly more important, physiological functions for the LOX pathway in plants remain to be discovered.

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